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Enterovirus Infections and Enterovirus Specific T-Cell Responses in Infancy

Sirpa Juhela,[1]* Heikki Hyöty,[1,2] Maria Lönnrot,[2] Merja Roivainen,[3] Olli Simell,[4] and Jorma Ilonen[1]

[1]*Turku Immunology Centre and Department of Virology, University of Turku, Finland*
[2]*Department of Virology, University of Tampere Medical School, Tampere, Finland*
[3]*Department of Virology, National Public Health Institute, Helsinki, Finland*
[4]*Department of Paediatrics, University of Turku, Finland*

The development of enterovirus specific T-cell and antibody responses were examined in a cohort of 60 healthy infants at the ages of 3, 6, 9, and 12 months. By the age of 6 months, 68% of the infants had developed T-cell responses against enterovirus antigens by lymphocyte proliferation test, whereas only 30% had serological evidence of an enterovirus infection. By this age, only 7% of the infants had adenovirus specific T-cell responses and 3% had serologically verified adenovirus infection. Enterovirus specific T-cell responses correlated with the lack of enterovirus antibodies in cord blood and the number of sibs reflecting protection by maternal antibodies and the rate of exposures, respectively. T-cell responses cross-reacted between different enterovirus serotypes. The results show that enterovirus infections occur frequently in infancy and induce T-cell immunity. Cellular immunity may be a more sensitive indicator of neonatal enterovirus infections than antibodies. ***J. Med. Virol. 54: 226–232, 1998.***

KEY WORDS: T-cells; lymphocyte proliferation; immunity.

INTRODUCTION

Enteroviruses are common human pathogens. Most enterovirus infections are subclinical or cause only mild respiratory symptoms, but severe complications such as myocarditis, meningitis, poliomyelitis, and life-threatening neonatal infections also occur occasionally [Abzug et al., 1993; Alexander et al., 1993; Berlin et al., 1993; Modlin et al., 1991]. Enterovirus infections may also play a role in the pathogenesis of certain autoimmune diseases, especially insulin-dependent diabetes mellitus and chronic cardiomyopathies [Dahlquist et al., 1995; Goren et al., 1989; Grist et al., 1978; Hyöty et al., 1995; Tracy et al., 1990]. Neonatal enterovirus infections are likely to be common, as maternal antibodies protect only from infections caused by the corresponding serotypes and cover only a portion of almost 70 different enterovirus serotypes [Abzug, 1995; Berry and Nagington, 1982; Dagan, 1996; Hammond et al., 1985]. The later protection against reinfection depends on circulating and mucosal neutralising antibodies [Dagan et al., 1983]. The cellular immune response is less well characterised, but it is probably important for the elimination of primary enterovirus infection and regulation of the immune response. The development of T-cell mediated immunity and antibody responses were evaluated in a prospective cohort of newborns. All infants were identical for HLA-DQB1 genotype. The effects of gender, age, maternal enterovirus antibodies in cord blood, duration of breast feeding, and the family size on the frequency of enterovirus infections and the cellular immune responses against enterovirus antigens were analysed.

MATERIALS AND METHODS

Study Design and Subjects

Study subjects were healthy children from general population who had HLA-DQB1*02/*0302 genotype associated with increased IDDM risk [Ilonen et al., 1996]. They were recruited from the Diabetes Prediction and Prevention (DIPP)-trial, newborns whose parents were willing to participate were screened and those with risk-associated genotypes were followed-up every 3 months with venous blood samples. Two to 5 ml of heparinised venous blood was collected at each control visit. A cohort of 60 infants was monitored at regular intervals up to 9 months and 39 of them up to 12 months.

Infants were immunised according to the standard

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*Correspondence to: Sirpa Juhela, Department of Virology, University of Turku, Kiinamyllynkatu 13, FIN-20520 Turku, Finland. E-mail: sirpa.juhela@utu.fi

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Finnish vaccination protocol including BCG immunisation of newborns at the age of few days and DPT (Diphtheria, Pertussis, Tetanus) vaccination at the age of 3, 4, and 5 months. Three infants had received the first DPT dose before 3 months sample was collected. Salk type of inactivated polio vaccine is given at the age of 6 and 12 months. All infants in the present study had received the first polio immunisation after the blood sample was taken at the age of 6 months and the second immunisation after collecting the 12 months' sample.

Lymphocyte Proliferation Assay

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation. The PBMC were washed and resuspended in RPMI 1640 medium supplemented with 10% human AB serum (Finnish Red Cross, Helsinki, Finland), glutamine, HEPES, and gentamycin 10 μg/ml. Fifty-thousand PBMC/well were incubated in quadruplicate with antigens in 200 μl final volume in 96-well round-bottomed microtitre plates for 6 days. Tritiated thymidine (2 μCi/ml, Amersham, UK) was added 18 hours before harvesting. The cultures were harvested on glass fibre filters using Tomtec 93 Mach III Manual Harvester (Tomtec, Orange, CT) and incorporated radioactivity was measured with Micro-Beta scintillation counter (Wallac, Turku, Finland). Stimulation indices (SI) were calculated by dividing the median cpm value of antigen-stimulated quadruplicate wells by the median cpm of quadruplicate control wells.

Antigens

Purified poliovirus types 1 and 3 and coxsackievirus B4 virions at 1 μg/ml and 0.1 μg/ml concentration were used to test proliferation responses against enteroviruses. Responses to adenovirus hexon protein (10 μg/ml and 1 μg/ml) [Waris and Halonen 1987], tetanus toxoid (TT; 1 μg/ml) [National Public Health Institute, Helsinki, Finland], and PPD (10 μg/ml; Statens Seruminstitut, Copenhagen, Denmark) were also studied. Pokeweed mitogen (12.5 μg/ml) was used as a mitogen control.

Virus Antibodies

Highly purified viruses were incubated at 56°C for 15 min prior to RIA or EIA analyses to expose antigenic determinants known to be cross-reactive with different enterovirus serotypes. IgG, IgM, and IgA class antibodies against coxsackieviruses B5 and A9 and echovirus types 1 and 11 were analysed using a heavy chain capture RIA and IgG class antibodies against coxsackievirus B3 and a synthetic enterovirus peptide using an indirect EIA method [Frisk et al., 1989; Hovi and Roivainen, 1993; Hyöty et al., 1995; Roivainen et al., 1993]. Serotype specific antibodies against coxsackievirus B serotypes 1 to 6 were studied using standard plaque neutralization assay, and IgG and IgA class antibodies against adenovirus hexon antigen (serotype 5) using an EIA [Hyöty et al., 1995]. Acute infection was diagnosed if antibody titers increased four-fold or greater or the sample contained IgM in amounts exceeding previously defined cut-off limit [Hiltunen et al., 1997; Hyöty et al., 1995].

Statistical Analysis

Two-tailed Mann-Whitney U-test and Student's *t*-test were used for comparison of two groups.

Ethics

The Joint Ethics Committee of the Turku University and Turku University Hospital had approved the DIPP project. Informed consent was obtained from all parents/guardians.

RESULTS

T-cell responses against the PPD and TT antigens followed closely the expected time table, as responses appeared according to the vaccination program of the infants (Table I). BCG immunisation, which was given on the first days of life generated strong responses to PPD antigen, and the responses remained almost stable at least to the age of 12 months, the end of this follow-up. T-cell responses to TT appeared first at the age of 6 months except in four cases, who responded already at the age of 3 months. A previous PDT vaccination was confirmed in three infants.

Proliferation responses to poliovirus types 1 and 3 were frequent already at the age of 3 and 6 months (Table I), although the infants had not been vaccinated with poliovirus vaccine by the time these samples were taken and no cases of natural poliovirus infections have occurred in Finland since 1986. The responses to polio antigens further increased after the polio vaccinations were given. Responses to coxsackievirus B4 (SI > 3) were detected at the age of 3 months in 35% (21/60) of the infants and at age of 6 months in 37% (22/60). A clear peak appeared at the age of 9 months as 58% (35/60) of the infants responded to coxsackievirus B4 (Table I). Strong T-cell responses (SI > 10) against coxsackievirus B4 were measured in 5%, 11%, 24%, and 5% of the infants at the ages of 3, 6, 9, and 12 months, respectively. The proliferation responses to polioviruses and coxsackievirus B4 correlated rather well, and the correlation became stronger when the polio immunisation with the Salk vaccine began at the age of 6 months (Fig. 1). Both genders showed similar T-cell proliferation responses against all the antigens studied.

Altogether 45 serologically confirmed enterovirus infections were found before 12 months age. The cumulative frequency of infections increased continuously, as 8%, 30%, 50%, and 60% of the infants had had at least one enterovirus infection by the ages of 3, 6, 9, and 12 months, respectively (Fig. 2). Twenty-five infants (42%) had a single enterovirus infection and 10 (17%) children had two or more infections during the follow-up. T-cell responses to coxsackievirus B4 at the age of 9 months, just after the polio immunisation,

TABLE I. The Proportion of Infants With Positive (SI > 3) and Strong Positive (SI > 10) T-Cell Responses

		Stimulation						
Age	Proliferation response	Pokeweed	PPD	Tetanus toxoid	Coxsackie B4	Polio type 1	Polio type 3	Adeno
3 months								
n = 60	SI > 3	60 (100%)	56 (93%)	4 (7%)	21 (35%)	35 (59%)	21 (35%)	7 (11%)
	SI > 10	60 (100%)	52 (87%)	1 (2%)	3 (5%)	9 (15%)	2 (3%)	1 (2%)
	median SI	164	70	1	2	3	2	1
6 months								
n = 60	SI > 3	60 (100%)	55 (91%)	41 (68%)	22 (37%)	37 (62%)	26 (44%)	4 (7%)
	SI > 10	60 (100%)	50 (84%)	29 (49%)	6 (11%)	16 (27%)	5 (9%)	0 (0%)
	median SI	112	60	10	2	3	3	1
9 months								
n = 60	SI > 3	60 (100%)	57 (95%)	46 (76%)	35 (58%)	43 (71%)	28 (47%)	6 (10%)
	SI > 10	60 (100%)	52 (87%)	31 (52%)	14 (24%)	27 (45%)	20 (34%)	0 (0%)
	median SI	112	60	12	4	9	6	1
12 months								
n = 39	SI > 3	39 (100%)	37 (95%)	28 (72%)	25 (64%)	28 (72%)	24 (62%)	2 (5%)
	SI > 10	38 (97%)	32 (82%)	20 (51%)	2 (5%)	11 (28%)	7 (18%)	1 (3%)
	median SI	110	55	10	3	7	5	1

*The median responses to various antigens at the age of 3, 6, 9, and 12 months are shown.

were stronger in infants who had had serologically documented enterovirus infection before the age of 6 months than in infants without previous infections (median SI 5.8 vs. 2.8; $P < 0.05$). Responses at other ages showed no such difference, and the proliferation responses to polioviruses were also stable irrespective of the previous serologically confirmed enterovirus infections.

Infants were also tested for serotype specific antibodies against all coxsackievirus B serotypes 1 to 6 using standard plaque neutralisation assay. Eight coxsackievirus B infections (six coxsackievirus B2 and two coxsackievirus B5) were found. One of these infections occurred between the ages of 3 and 6 months, five infections between 6 and 9 months, and two infections between 9 and 12 months. Thus, the majority of the 45 serologically documented enterovirus infections were caused by other enteroviruses than the coxsackie B viruses. Those infants who according to the plaque neutralisation assay had experienced coxsackievirus B infections had also stronger T-cell responses to coxsackievirus B4 than the other children (median SI 10.8 vs. 4.2 at age of 9 months; $P < 0.05$; Fig. 3), even though strong responses to coxsackievirus B4 were also measured in children who became infected by other enteroviruses.

T-cell proliferation responses against enterovirus antigens were markedly stronger in infants who had no IgG enterovirus antibodies in cord blood than in infants who had antibodies in cord blood. At the age of 6 months the median SIs against coxsackievirus B4 and type 1 and 3 polioviruses were 2.9, 11.9, and 5.7 in cord blood antibody negative infants and 1.4, 2.2, and 2.3 in cord blood antibody positive infants, respectively ($P < 0.05$). Five infants had IgA class enterovirus antibodies in cord blood. These infants had stronger T-cell responses to coxsackievirus B4 than the other infants at the age of 3 months (median SI 3.0 vs. 1.1; $P < 0.05$). Duration of exclusive or partial breast feeding showed no correlation with T-cell responses against the enterovirus antigens.

T-cell responses against enterovirus antigens were clearly linked with the number of children in the family. Infants with one or more siblings had significantly higher T-cell responses to type 1 and 3 poliovirus and to coxsackievirus B4 at 3 months of age (median SIs 2.4, 4.3, and 2.4, respectively) than single infants (1.4, 2.8, and 1.8, for each antigen P for difference <0.05). According to serology the infants with siblings had also experienced slightly more enterovirus infections before the age of 6 months (12/32; 38%) than single infants (5/25; 20%).

Only 11% of children had T-cell proliferation responses (SI > 3) to adenovirus antigen during the entire follow-up (Table I) and serology verified adenovirus infections in only 10 infants. Two of the infections occurred between the ages of 3 and 6 months, four between 6 and 9 months, and four between 9 and 12 months (Fig. 2). A serologically confirmed adenovirus infection also associated with stronger T-cell responses to adenovirus in the children (median SI 3.2 in children with serologically confirmed adenovirus infections vs. 1.0 in children without serological evidence of adenovirus infection; $P < 0.001$).

DISCUSSION

This study shows that most infants develop T-cell responses against enterovirus antigens during the first 6 months of life, whereas serological evidence of enterovirus infection was found in only 30% of the infants using group specific antibody assays. This indicates that lymphocyte proliferation test is a more sensitive measure of sensitisation than the current antibody assays.

All children in this study share the same HLA-DQB1 genotype, which might effect the immune responses against enteroviruses. This particular genotype carrying a strongly increased risk of IDDM combines DR3

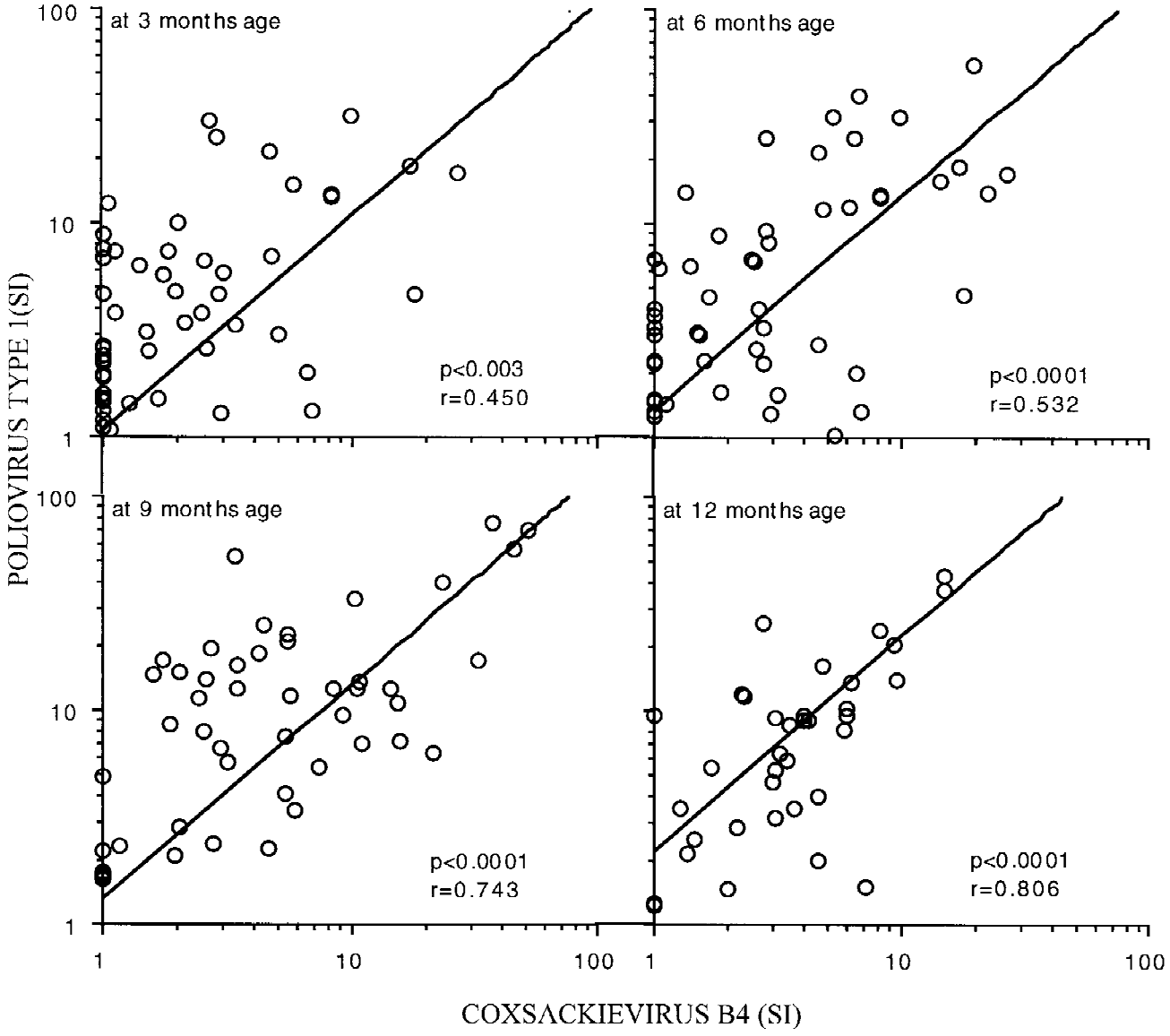

Fig. 1. Correlation between T-cell responses (in SI) to coxsackievirus B4 and poliovirus type 1 at the ages of 3, 6, 9, and 12 months in a cohort of 60 newborns, who were followed from birth to 9 months and in 39 of these children who were followed to the age of 12 months.

associated DQB1*02 and DR4 associated DQB1*0302. These two haplotypes have been reported to be associated with either low (DR3) or high (DR4) responsiveness to enterovirus antigens [Bruserud et al., 1985a,b]. Heterozygosity for both haplotypes as was the case in the present series produces an intermediate level of responsiveness. This has also been observed in our own studies [Juhela et al., unpublished material] suggesting that the findings of the present study are quite representative for the general population as well.

Typical year-around rates of adenovirus and enterovirus infections were observed in Finland during the study period. The difference in the number of adenovirus and enterovirus infections thus suggests that the difference in rate is a true phenomenon characteristic for this age. Such difference in infection rates may be due to more frequent exposure of the infants to enteroviruses than adenoviruses, or to a poor protection of an infant by maternal enterovirus antibodies. As protection by maternal antibodies is serotype specific and the number of enterovirus serotypes is high, the infants are probably protected against only some of them [Dagan, 1996]. The "leaky" protection is further supported by our study in which antibodies against CBV serotypes 1 to 6 were observed in 40%, 62%, 56%, 66%, 55%, and 24% of pregnant mothers, respectively, when plaque neutralisation test was used [Lönnrot et al., unpublished material].

Quite uniquely, the first polio vaccination is given at the age of 6 months in Finland. Such timing gave us an opportunity to study the induction of T-cell responses during natural enterovirus infections which occurred before 6 months of age, as well as to analyse immunological responses induced or enhanced by the killed

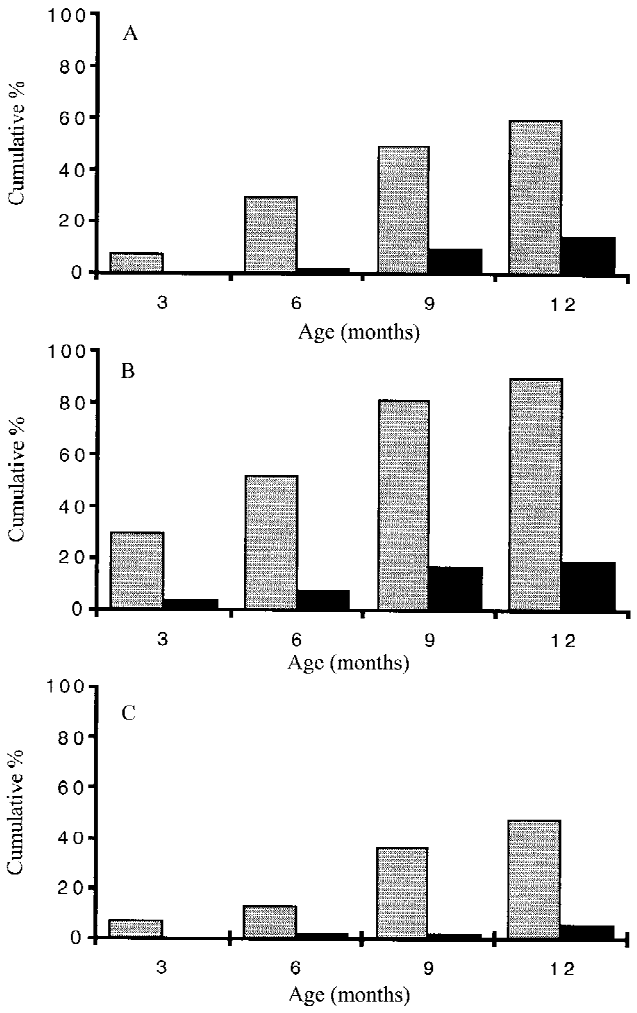

Fig. 2. Cumulative frequency of **(A)** serologically documented enterovirus and adenovirus infections as well as **(B)** positive (SI > 3), and **(C)** high positive (SI > 10) T-cell responses to coxsackievirus B4 and adenovirus antigens in a cohort of 60 newborns. The whole cohort was followed until the age of 9 months and 39 of them until the age of 12 months (Grey bars: enterovirus/coxsackievirus B4; Black bars: adenovirus).

vaccine. As all infants received their first polio vaccination between the ages of 6 and 9 months, T-cell responses against polioviruses were markedly stronger at the age of 9 months than before. A simultaneous increase occurred also in responses against coxsackievirus B4. The wide cross reactivity of the T-cell responses against various enterovirus serotypes was clearly demonstrated by the occasionally strong poliovirus responses which were measured before the infants were vaccinated against polio. Live attenuated poliovirus (oral) vaccination has not been used in Finland during the past 10 years, thus excluding the possibility of contacts with vaccine strains. Meanwhile, T-cell responses against coxsackievirus B4 were often strong in children with no antibodies to this serotype, and in coxsackievirus B2 and B5 infections confirmed by neutralisation test several infants showed strong responses against polioviruses. After polio vaccination T-cell responses to coxsackievirus B4 were also stronger in children who had had previously enterovirus infections indicating activation of coxsackievirus B4 specific memory T-cells by the vaccination. T-cell responses against polioviruses correlated also with T-cell responses against coxsackievirus B4.

T-cell cross-reactivity was an expected phenomenon as different enterovirus serotypes contain several consensus regions, which probably also serve as T-cell epitopes [Cello et al., 1996; Mahon et al., 1992]. Healthy adult volunteers show lymphocyte proliferation responses to several enteroviral antigens, but these responses correlate poorly with serotype-specific immunity i.e. neutralising antibodies [Beck and Tracy, 1990], indicating that T cells indeed recognise cross-reactive epitopes widely. T-cell lines generated by Graham et al. [1993] further demonstrated this cross-reactivity when various enterovirus antigens were used.

T-cell proliferation assays thus appear to be more sensitive indicators of immune activation against enterovirus antigens than the conventional antibody assays. This sensitivity may be due to the fact that immune responses against several enterovirus serotypes were found frequently in T-cell proliferation tests whereas current antibody assays obviously detected fewer serotypes. Serotype restriction of antibody tests make them insensitive in the diagnosis of enterovirus infections, and the humoral immune responses vary markedly between individuals [Danes et al., 1983]. For example, IgM responses develop in only 40–60% of the patients. The findings imply that serological tests alone underestimate the number of enterovirus infections. Interestingly, the T-cell responses declined usually within 3 months suggesting that in infants strong proliferation responses (i.e., SI > 10) probably indicate that the subject has recently had enterovirus infection or received polio vaccination.

The frequency of enterovirus infections in infants depends on the number and intensity of exposures and on protective immunity which is due mainly to transplacentally acquired maternal antibodies or to antibodies received in breast milk. Infants with sibling(s) had more enterovirus infections and stronger T-cell responses to enterovirus antigens than the single infants probably indicating more enterovirus exposures in families with several children. Enterovirus infections and enterovirus specific T-cell responses were also more common in infants who had no maternal IgG enterovirus antibodies in the cord blood than in infants who were antibody positive. Previous studies have shown that breast feeding decreases the number of enterovirus infections in the infant [Beaudry et al., 1995; Jenista et al., 1984]. However, the effect of breast feeding on the protection from enterovirus infections could not be reliably evaluated in the present study, because almost all infants were breast fed; 95% of the infants began to receive breast milk after birth, and 81% were still receiving solely breast milk at the age of 3 months.

In conclusion, this study demonstrates that entero-

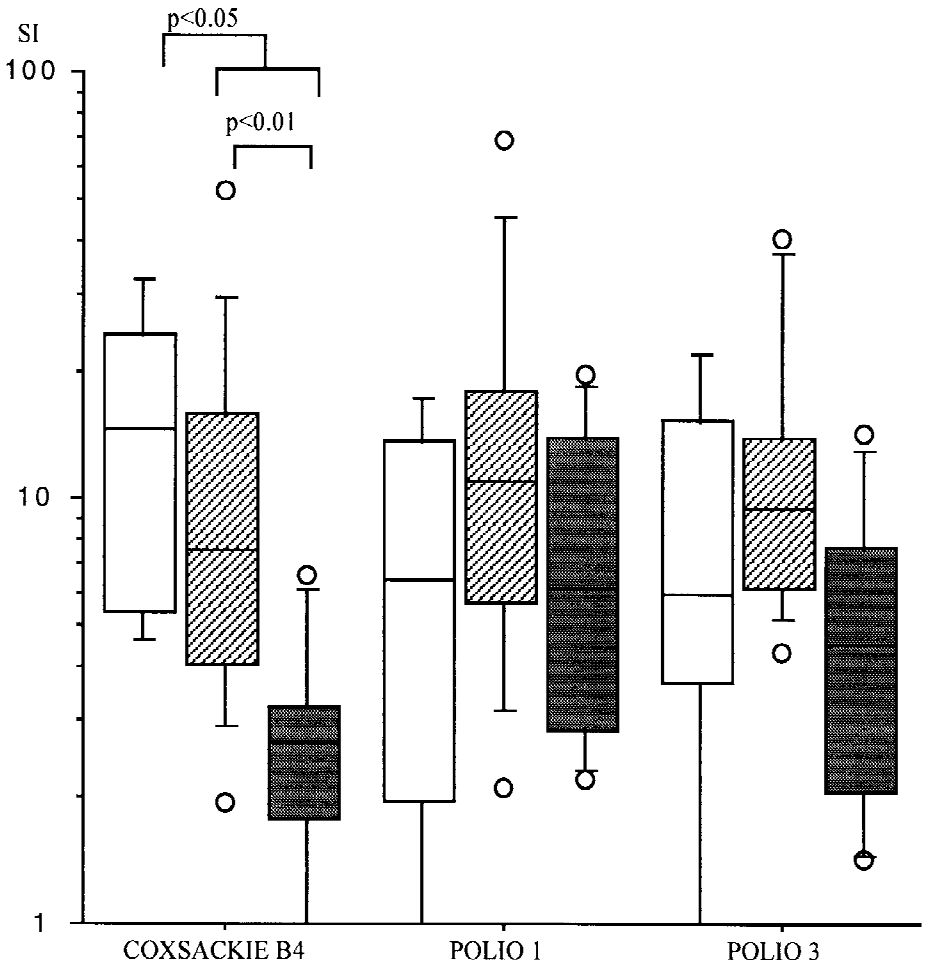

Fig. 3. T-cell responses against enterovirus antigens at the age of 9 months in infants with coxsackie B infection measured using neutralisation assay (white bars), in children infected by other enterovirus serotypes (hatched bars), and in children without serological evidence of enterovirus infection (grey bars) by the age of 9 months.

virus infections are frequent in young infants, these infections induce strong T-cell responses and that cellular immune responses may be more sensitive indicators of recent enterovirus infections than conventional group specific antibody assays. T-cells showed wide cross-reactivity between enterovirus serotypes, which may also have clinical importance in the protection against enterovirus infections [Tracy et al., 1995].

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